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Acute lung injury (ALI) related to sepsis activates tissue remodeling that is responsible for the excessive deposition and turnover of extracellular matrices. This project will explore the factors that control lung tissue remodeling in the setting of sepsis by focusing on chronic ethanol ingestion, a factor that renders the lung susceptible to ALI. We hypothesize that chronic ethanol ingestion renders the lung susceptible to ALI by acting on $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChRs) and stimulating the expression of tissue remodeling genes such as that of fibronectin (FN). Aberrant deposition of FN affects the structure of the lung and promotes a "proinflammatory state" that drives the development of ALI after infection. The specific aims are to: 1) Determine the role of $\alpha 7$ nAChRs in ethanol induction of FN. 2) Delineate the intracellular pathways responsible for the induction of FN in fibroblasts in response to ethanol. 3) Elucidate the effects of ethanol-induced FN expression on lung cell function. 4) Study ethanol-induced FN expression in a rat model of sepsis-induced ALI. Service men and women are exposed to conditions considered risk factors for ALI (e.g., trauma, toxic gas, infection). Tissue remodeling is considered key to the development of the irreversible consequences of ALI.

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I. INTRODUCTION

Acute lung injury is a major cause of morbidity and mortality in the U.S. Its most dramatic manifestation is the Acute Respiratory Distress Syndrome or ARDS, an illness that affects over 150,000 Americans each year and that leads to death in up to 40-50% of cases (1). Acute lung injury is characterized by the activation of tissue remodeling which is responsible for the excessive deposition and turnover of extracellular matrices (2). Ultimately, it is the ability of the host to control tissue remodeling that determines the final outcome in acute lung injury (2,3). Although external factors capable of eliciting acute lung injury have been identified (e.g., infection, trauma), little is known about the factors that control the tissue remodeling response. This project addresses this very aspect. It was prompted by an intriguing report published in 1996 linking chronic ethanol ingestion to outcomes in ARDS (4). This report identified ethanol as an independent outcome variable in ARDS, a finding that is considered one of the most significant observations made in the area of acute lung injury. Today, it is believed that the development of acute lung injury is related to chronic ethanol ingestion in over 50% of cases (5). Because of its importance, we began to explore the mechanisms by which ethanol affects tissue remodeling and predisposes the lung to acute lung injury. Preliminary observations made in this area led us to hypothesize that ethanol ingestion renders the lung susceptible to acute lung injury by acting on $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) expressed by fibroblasts, and stimulating their expression of tissue remodeling genes; in particular that of fibronectin (FN). The consequent aberrant deposition of FN in the lung parenchyma induces the expression of potent transcription factors (e.g., AP-1, NFkB) in macrophages and other cells that come in contact with the newly deposited fibronectin-containing matrix. This promotes a "proinflammatory state" that primes resident and incoming immune cells recruited by diverse pulmonary insults (e.g. infection) thereby amplifying inflammatory responses in the lung that promote the development of acute lung injury. The following objectives were designed to address the hypothesis:

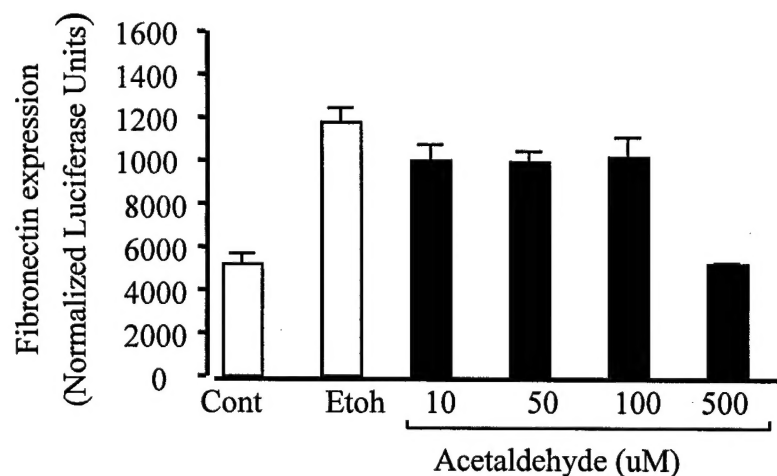
- Objective I. Determine the role of $\alpha 7$ nAChRs (and perhaps other nAChRs) in ethanol induction of FN.
- Objective II. Delineate the intracellular pathways responsible for the induction of FN in fibroblasts in response to ethanol.
- Objective III. Elucidate the effects of ethanol-induced FN expression on cytokine expression.
- Objective IV. Study the effects of ethanol-induced FN expression in the rat model of sepsis-induced acute lung injury.

II. BODY

This work has led to important observations that are described below under each of the objectives proposed in the initial application:

Objective I. Determine the role of $\alpha 7$ nAChRs (and perhaps other nAChRs) in ethanol induction of fibronectin.

During the first year of this project, we found that ethanol stimulates transformed and primary lung fibroblasts to express fibronectin mRNA and protein. These effects appeared to be mediated via $\alpha 7$ nAChRs and required protein kinase C activation and DNA binding by the transcription factor CREB. This work has been submitted to the *American Journal of Physiology* for publication and is currently under revision. In the period related to the current report, we attempted to prove if indeed ethanol affects fibroblast through $\alpha 7$ nAChRs. To this end, we obtained mice with a knockout mutation in $\alpha 7$ nAChRs. Lungs from these animals were processed and primary fibroblastic cell lines were created. Using these lines, we have found that nicotine does not affect fibronectin expression indicating that they indeed lack $\alpha 7$ nAChRs. Currently, we are in the process of testing the effects of ethanol in these cells. In other work, we have explored the role of ethanol metabolism in our system. We found that 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, inhibited ethanol-induced fibronectin expression in fibroblasts. This suggests that the main player in this process is acetaldehyde. To test this, we exposed cells to acetaldehyde and found that this molecule indeed stimulated fibronectin expression (see figure below).



The latter observation suggests that lung fibroblasts contain alcohol dehydrogenase and that metabolism through this enzyme is required to allow for the effects of ethanol on fibronectin expression.

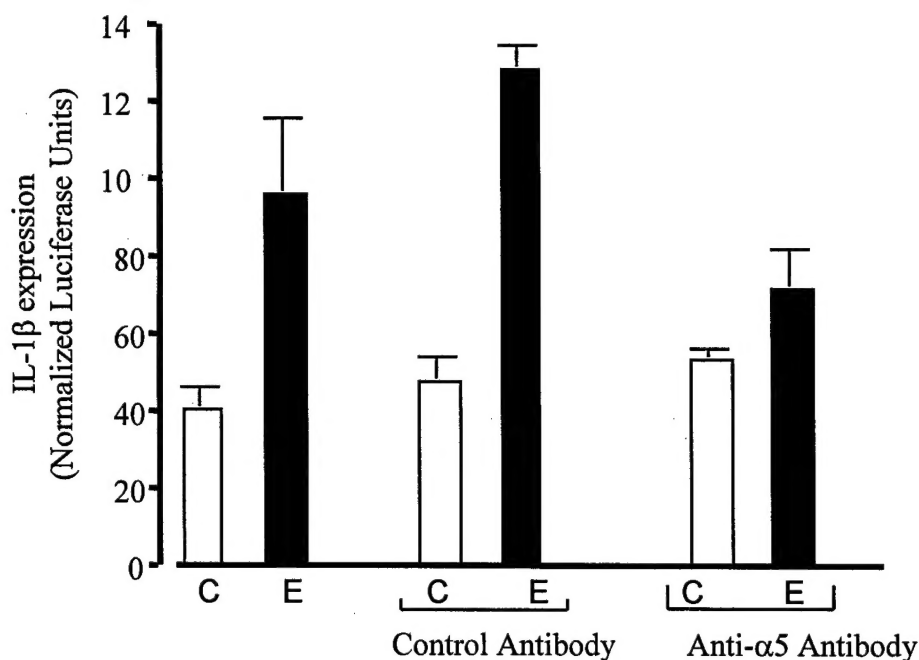
Objective II. Delineate the intracellular pathways responsible for the induction of fibronectin in fibroblasts in response to ethanol.

In the previous year, we determined that ethanol stimulates fibronectin expression in fibroblasts by stimulating the transcription of the fibronectin gene. We delineated a number of signaling pathways and the transcription elements on the fibronectin gene promoter that were involved in this process.

In the period of this report, we investigated if endotoxin was capable of stimulating fibronectin expression. This is important because our hypothesis suggests that ALI in alcoholics is triggered by a second hit; infection representing a common example of this. In fact, sepsis is the most common cause of ALI in the U.S., and it is often the consequence of infection with gram negative bacteria (e.g., pseudomonas) that release endotoxins. We failed to detect a change in fibronectin expression in response to endotoxin. However, we found that endotoxin was capable of stimulating human U937 monocytic cells to express a fibronectin receptor, the integrin $\alpha 5 \beta 1$. This is an important finding because it suggests that endotoxemia during sepsis might promote increased recognition of fibronectin matrices by monocytic cells through induction of fibronectin receptors. In turn, this would facilitate cellular migration into tissues with subsequent damage upon activation. We found that the lipid A portion of endotoxin was most responsible for this effect and that it depended on CD14 expression on the surface of the cells. Through induction of protein kinase C activation and DNA binding by the transcription factor Nf κ B, endotoxin induced the transcription of the gene encoding for the $\alpha 5$ subunit of the $\alpha 5 \beta 1$ integrin. These changes were associated with increased adhesion to fibronectin. This work was accepted for publication in the *American Journal of Physiology*.

Objective III. Elucidate the effects of ethanol-induced fibronectin expression on cytokine expression.

We are pleased to announce that we have begun to work in this area. Our hypothesis suggests that excessive deposition of fibronectin in lung may alter the behavior of immune cells recruited to the lung. To test this possibility, we harvested alveolar type II cells from rats exposed to ethanol for 6 weeks and cultured them for up to 3 days thereby allowing the cells to deposit an insoluble matrix. At the end of the culture period, the cells were eradicated and the remaining matrix-coated plates were used for further experiments. Human monocytic U937 cells were cultured on the matrices followed by testing for expression of the pro-inflammatory cytokine interleukin-1 β (IL-1 β). To facilitate the detection of IL-1 β expression, U937 cells were stably transfected with a construct containing the human IL-1 β promoter fused to a luciferase reporter gene. The U937s were allowed to settle on the primary cell-derived matrices for 24-48 hours followed by collection of the cells and luciferase detection. As depicted in the figure below, we found that cells cultured on matrices derived from cells harvested from ethanol-treated animals (E) produced more IL-1 β than those cultured on matrices derived from control animals (C). Furthermore, we found that an antibody to $\alpha 5 \beta 1$ integrin inhibited the response, whereas a control antibody did not. This observation suggests that the matrices deposited in the lung in alcoholics might affect the behavior of incoming monocytic cells and could promote a pro-inflammatory state. They also suggest that fibronectin receptors play a key role in the recognition of these fibronectin-containing newly produced matrices



Objective IV. Study the effects of ethanol-induced fibronectin expression in the rat model of sepsis-induced acute lung injury.

In the first year of this project, we found that the lungs of rats that were fed the Lieber-DeCarli isocaloric liquid diet that contains 36% of total calories provided as ethanol, showed increased accumulation of fibronectin mRNA (by RT-PCR) and protein (by immunohistochemistry). We have also begun to explore other tissue remodeling molecules in the alcoholic lung as it relates to sepsis. This work has led us to study Matrix Metalloproteinase-9 (MMP-9), a matrix degrading protease that has been implicated in ALI. Because fibronectin can stimulate MMP-9 expression, we studied MMP-9 in the lungs of rats exposed to ethanol for 6 weeks. We found that ethanol alone did not induce MMP-9 mRNA and activity (as determined by gelatin zymography). However, in the presence of endotoxemia, it did. We have recapitulated these findings in cultured human monocytic THP-1 cells. When cultured in the presence of ethanol (60 nM), these cells show basal level expression of MMP-9. However, addition of endotoxin (5 ug/ml) induced a dramatic increase in MMP-9 mRNA and activity. This effect was blocked by inhibitors of protein kinase C. This work suggests that chronic alcohol abuse and endotoxemia might interact to stimulate tissue remodeling through a number of pathways that include the increased deposition of connective tissue (e.g., fibronectin), while stimulating its degradation. We predict that such scenario would lead to alterations in matrix composition in the lung rendering it susceptible to injury.

In addition to the above, with Dr. David Guidot (collaborator), we have established the sepsis model in rats since this is the key component of this objective. This model requires opening the rat abdomen and ligating the cecum followed by closure. In addition, we have spent a lot of time in generating the tools necessary to perform the work proposed. In this regard, we have obtained more experience with the imaging and morphometric analysis tools that will be used for analysis of lung structures. We have also generated a number of RNA probes to be used

in RT-PCR experiments designed to study the effects of alcohol on the expression of cytokines and other pro-inflammatory molecules in the sepsis model. In addition, we have established a collaboration with Dr. Mauricio Rojas (Assistant Professor, Division of Pulmonary Medicine, Emory University) who will assist us in detecting cytokine proteins in lung fluids using the Luminex system. We will be focusing on this model and how it relates to chronic ethanol exposure and lung tissue remodeling for the next 2 years of the project. A revised statement of work is being prepared to this effect.

III. KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that ethanol stimulation of fibronectin requires metabolism through alcohol dehydrogenase.
- We obtained $\alpha 7$ nAChR knockout animals to study the role of this receptor in ethanol-mediated effects in the lung.
- We showed that alterations in the composition of cell matrices, with relative increases in fibronectin content, stimulate human monocytic cells to express proinflammatory cytokines such as IL-1 β .
- We have found that endotoxin, a product of sepsis with gram negative bacilli, increases the recognition of fibronectin matrices in monocytic cells by stimulating the expression of functional fibronectin $\alpha 5 \beta 1$ receptors.
- We have found that ethanol and endotoxin work together to stimulate the expression/activity of a matrix degrading enzyme called MMP-9. This suggests that chronic ethanol abuse might promote tissue remodeling through effects on several connective tissue molecules.
- We have established the model of sepsis in rats, we have generated experimental reagents, and we have obtained expertise with the methodology required to address our hypothesis in the experimental model.

IV. REPORTABLE OUTCOMES

- **Abstracts/poster presentations**

Roman J, Ritzenthaler JD, Guidot DM, Brown LA. Ethanol induces alveolar type II cells to deposit a matrix that promotes monocyte activation. Poster to be presented at the 27th annual Research Society of Alcohol Meeting to be held in Canada in 2004.

Tomic R, Ritzenthaler JD, Roser S, Guidot DM, Roman J. Matrix metalloproteinase-9 expression in chronic ethanol abuse and endotoxemia-related acute lung injury. Poster to be presented during the upcoming American Thoracic Society Meeting to be held in May 2004 in Orlando, Florida.

- **Manuscript in press or submitted for publication in peer-reviewed journals**

Roman J, Ritzenthaler JD, Boles B, Lois M, Roser-Page S. Lipopolysaccharide induces the expression of fibronectin $\alpha 5\beta 1$ integrin receptors on human monocytic cells in a Protein Kinase C-dependent fashion. *Am J Physiol*, in press.

Roman J, Ritzenthaler JD, Bechara R, Brown LA, **Guidot DM**. Ethanol stimulates the expression of fibronectin in lung fibroblasts via nicotinic acetylcholine receptor-dependent signals. *Am J Physiol*, under revision.

Bechara RI, Brown LA, Eaton DC, **Roman J**, **Guidot DM**. Chronic ethanol ingestion increases expression of the angiotensin II type 2 (AT2) receptor and enhances tumor necrosis factor- α - and angiotensin II-induced cytotoxicity via AT2 signaling in rat alveolar epithelial cells. *Alcohol Clin Exp Res*. 2003 Jun;27:1006-14 (included as submitted in first year's report).

- **Presentations**

Dec 2003 **Guidot DM**: Lecture, *Effects of alcohol in the lung during sepsis*. Department of Medicine Research Seminar at Emory University.

Feb 2004 **Roman J**: Lecture, *Ethanol, endotoxemia, and fibronectin expression in the lung*. Emory Pulmonary Grand Rounds.

Apr 2004 **Roman J**: Lecture: *Ethanol, acetylcholine receptors, and fibronectin expression: role in acute lung injury*. Department of Defense Symposium to be held in San Juan, Puerto Rico.

V. CONCLUSIONS

Our data suggest that ethanol predisposes subjects to ALI during sepsis by stimulating lung tissue remodeling characterized by increased expression of fibronectin and MMP-9. The newly deposited matrices can stimulate incoming monocytic cells to express increased amounts of pro-inflammatory cytokines such as IL-1 β . These incoming cells recognize fibronectin matrices through endotoxin-induced upregulation of surface fibronectin $\alpha 5\beta 1$ integrin receptors. This work is tantalizing, but the hypothesis needs to be tested in vivo. In discussions with the DOD, and due to overlap with an NIH funded project, we plan to focus exclusively on work related to the sepsis model as described in Objective IV of the original proposal for the remainder of the project.

VI. REFERENCES

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